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Engineering monomeric streptavidin and its ligands with infinite affinity in binding but reversibility in interaction

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ABSTRACT

Natural tetrameric streptavidin captures and immobilizes biotinylated molecules with ultra-tight binding ($K_d \sim 10^{-13}$ to 10^{-14} M). In contrast, engineered monomeric streptavidin offers reversible binding ($K_d \sim 10^{-7}$ M). To develop an ideal engineered streptavidin which possesses both the immobilization capability of the natural streptavidin and the reversible interaction reactivity of the monomeric streptavidin, a pair of engineered biomaterials was designed through molecular modeling. This system consists of two recombinant components: an engineered monomeric streptavidin M6, which has a cysteine residue (C118) near the biotin binding site, and a cysteine containing biotinylation tag. Interactions between M6 and the biotinylated peptide tag go through a two-stage process (capture and immobilization) to generate a covalently linked complex. Biotinylation is essential in the capture stage. Once the biotin moiety in the biotinylated tag is captured by M6, the biotinylated tag can fold back and rotate on the surface of the complex with the biotinylated lysine in the peptide tag as the axis until the formation of a disulfide bond. Consequently, cysteine residue in different positions flanking the biotin residue in the biotinylation tag can successfully form a disulfide bond with M6. Intermolecular disulfide bond formation between M6 and the tag containing protein offers the immobilization capability to M6. In the presence of reducing agent and biotin, bound ligands can be dissociated. This system has the potential to extend the biotin-streptavidin technology to develop reusable biosensor/protein chips and bioreactors.

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Key words: biotin; biotinylation tag; immobilization; cysteine; disulfide bond; biotin ligase; *Bacillus subtilis*; secretion; staphylokinase; protein engineering.

INTRODUCTION

Streptavidin is a homotetramer with a biotin binding site in each subunit. The tight binding of streptavidin to biotin ($K_d \sim 10^{-13}$ to 10^{-14} M),[1,2] while allowing the use of streptavidin as an excellent capturing and immobilization agent in many applications,[3] suffers the drawback of being essentially irreversible in nature. To extend the applications of the streptavidin technology, engineered monomeric streptavidin molecules with reversible biotin binding capability have been developed.[4,5] One of them, designated M4, carrying four mutated residues,[5] is developed on the basis that a complete biotin binding pocket requires the donation of a tryptophan residue (Trp-120) from the neighboring subunit. M4, because of its monomeric nature, has an incomplete biotin-binding pocket and hence can bind biotin reversibly ($K_d \sim 10^{-7}$ *M*). M4 has been shown to be a good candidate for affinity purification of biotinylated biomolecules,[6] but it is not suitable for the immobilization of these molecules. In this study, we have developed a novel version of monomeric streptavidin, designated M6, which retains the reversible biotin binding capability yet at the same time has the immobilization capability. Although reversible binding and immobilization seem to be contradictory features that cannot be combined together, we present here a solution. A cysteine residue is introduced around the biotin binding pocket in M6. As well, a cysteine containing peptide tag for biotinylation (CPFB) is constructed. This 15 amino-acid peptide tag[7,8] can be enzymatically biotinylated using the *E. coli* biotin ligase, BirA.[9,10] Transient capturing of biotinylated CPFB by M6 will possibly position the cysteine residue in the CPFB tag very close to the cysteine residue in M6. Formation of a disulfide bond between these two interacting molecules converts the transient binding interaction into

Abbreviations: BCAP, biotinyl ε-aminocaproic acid; BirA, biotin liagase; BSA, bovine serum albumin; CPFB, cysteine containing peptide tag for biotinylation; M4, monomeric streptavidin (carrying 4 mutations in reference to the wild type streptavidin sequence); M6, monomeric streptavidin (carrying 6 mutations in reference to the wild type streptavidin sequence); ME, β-mercaptoethanol; PBS, physiological buffered saline; SAK, staphylokinase; SAK-CPFB, staphylokinase fusion with a C-terminal cysteine containing peptide tag for biotinylation; SAV, streptavidin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SELDI-TOF-MS; surface-enhanced laser desorption/ionization time-of-flight mass spectrometer; TCEP, tris(2-carboxyethyl)phosphine.

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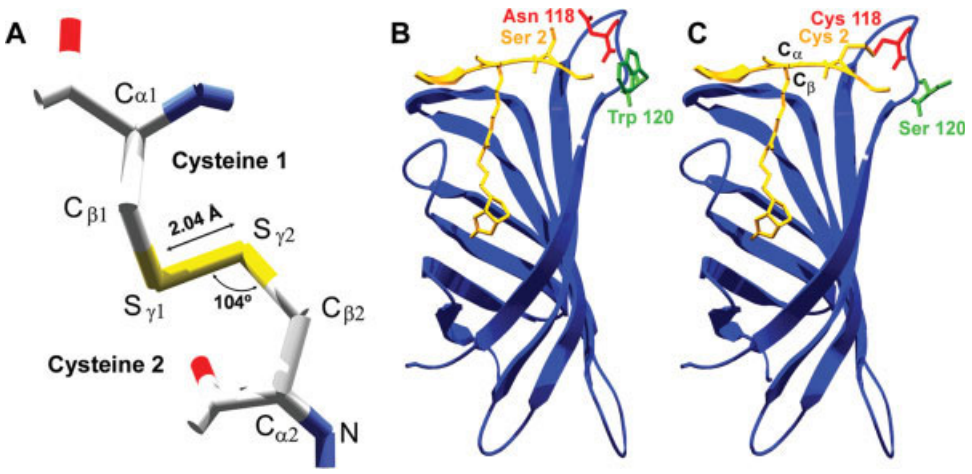

Figure 1

Engineering of monomeric streptavidin M6 and biotinylated peptide tag. (**A**) Steric requirements for the formation of disulfide bonds. (**B**) Model of a nonapeptide bound to a streptavidin subunit. The lysine residue at the center of this nonapeptide is biotinylated. Key residues in both streptavidin (blue) and the nonapeptide tag (yellow) subject to site-directed mutagenesis are shown. (**C**) Model of the cysteine containing peptide tag bound to M6. The intermolecular disulfide bond was generated by the use of the "Disulfide by Design" program.

covalent bond formation with infinite interaction affinity. Addition of reducing agents and biotin reverses the binding. The combination of these two engineered biomaterials offers many potential applications including development of reusable sensor chips and enzyme bioreactors, quantification of biotinylated cell surface receptors without inducing cross-linking and affinity purification of recombinant proteins.

MATERIALS AND METHODS

Modeling of streptavidin-peptide complexes

The coordinates of the streptavidin: biotinyl ε-aminocaproic acid (BCAP) complex (PDB 1LCZ)[11] were used to generate the structure of the tetramer by applying crystallographic symmetry operators. Serine nonapeptides in which the central or fifth serine residue was replaced with lysine were generated using the program CNS[12] [Fig. 1(B)]. All backbone torsion angles were set to ideal values for an extended β-strand conformation. The lysine residue of the nonapeptide was superimposed onto one of the BCAP residues of the streptavidin-BCAP tetramer using Xfit.[13] Different peptide orientations were generated by adjusting the torsion angles of the aliphatic portion of the biotinylated lysine residue.

Construction of an expression vector for the production of M6

Using pV55T-T76R-L109T-V125R,[5] the *B. subtilis* expression vector producing monomeric streptavidin M4 (T76R, V125R, V55T, L109T) as the template and BSSACBF (5′-CAAGCAACAGTATTAACC) and SAVNWB (5′-GTAGTACTTTTTGATGCGCATGCTTCTGTTGTTCC AGATG-3′) as the primers, two additional point mutations were introduced to the coding sequence of M4 by PCR-based oligonucleotide-directed mutagenesis. The amplified product was digested with *Hin*dIII and *Sca*I and used to replace the corresponding fragment in pV55T-T76R-L109T-V125R to produce pUB18-M6 (carrying T76R, V125R, V55T, L109T, N118C, and W120S mutations).

Construction of pSAK-CPFB

pSAKPFB is a pWB980-based vector[14] for secretory production of staphylokinase (SAK) containing a C-terminal biotinylation tag (PFB).[7] Different derivatives of pSAKPFB were constructed by PCR-based oligonucleotide-directed mutagenesis, using pSAKPFB as the template and a set of mutagenic primers listed in Table I. These primers introduced a cysteine residue at different positions relative to the lysine residue on the PFB tag (see Fig. 2). The amplified products were digested with the appropriate set of restriction enzymes and used to replace the corresponding fragment in pSAKPFB to generate pSAK-CPFB vector series.

Production and purification of M6 and SAK-CPFB

M6 and SAK-CPFB were prepared from the culture supernatants of *B. subtilis* WB800 harboring the appropriate vectors.[15] M6 was purified to homogeneity using the procedures used for M4 purification[5] with a signifi-

Table I
Mutagenic Primers for the Construction of SAK-PFB Derivatives

SAK-CPFB (+1) (*Xho*I/*Cla*I)	
Forward primer	BSSACBF
	5′-CAAGCAACAGTATTAACC-3′
Backward primer	PFB(+1)B
	5′-CTCTATCGATGATTCCAAAC**GCA**TTTTTGTGCATC-3′
SAK-CPFB (+2) (*Xho*I/*Cla*I)	
Forward primer	BSSACBF
Backward primer	PFBCYS(+2)B
	5′-CTATCGATGATTCCA**GCA**CATTTTTTGTGCATC-3′
SAK-CPFB (+4) (*Xho*I/*Cla*I)	
Forward primer	BSSACBF
Backward primer	PFBCYS(+4)B
	5′-GGAAGCTTATCGATG**GCA**CCAAACCATTTTTTGTG-3′
SAK-CPFB (−1) (*Bam*HI/*Nco*I)	
Forward primer	PFBCYS (−1)F
	5′-TTGGATCCCTTCATCATATTCTTGA**TGC**ATGCAAAA TGGTTTGG-3′
Backward primer	PUB19NCOB
	5′-CAAGAATATGCAGAAGTTCAAAGTAATC-3′
SAK-CPFB (−2) (*Xho*I/*Cla*I)	
Forward primer	BSSACBF
Backward primer	PFBCYS(−2)B
	5′-CTATCGATGATTCCAAACCATTTTTTG**GCA**ATCAA GAATATGATG-3′
SAK-CPFB (−6) (*Spe*I/*Nco*I)	
Forward primer	PFBCYS(−6)F
	5′-GTGGATCTACTAGTGGCTCTGGATCCCTTCAT **TGC**ATTCTTGATGCA-3′
Backward primer	PUB19NCOB
SAK-PFBC(−10) (*Spe*I/*Nco*I)	
Forward primer	PFBCYS(−10)F
	5′-CAAGTGGTGGATCTACTAGTGGCTCT**TGC**TCCCTT CATCATATTC-3′
Backward primer	PUB19NCOB
SAK-CPFB (−12) (*Spe*I/Nco*I*)	
Forward primer	PFBCYS(−12)F
	5′-CTGGTGGGTCGACAAGTGGTGGATCTACTAGT **TGC**TCTGGATCCCTTCATC-3′
Backward primer	PUB19NCOB

Mutated codons are bolded and underlined. Restriction enzymes in parentheses after the mutants refer to the set of enzymes used for cloning.

cant modification. A low salt buffer (10 m*M* sodium phosphate, pH 6.8, 10 m*M* sodium chloride) was used throughout the purification procedure. Unbiotinylated SAK-CPFB was purified on a Macro Q column (GE Healthcare) according to Szarka *et al.*[16] Biotinylation of SAK-CPFB was achieved by using *E. coli* engineered biotin ligase (CBD-BirA)[7] and the biotinylated proteins were purified on a monomeric avidin agarose column.[17] Purified proteins were quantified by their absorbance at 280 nm using their respective molar extinction coefficients.[18]

Kinetic analysis of M6 streptavidin mutein

The kinetic parameters (both on- and off-rates for interaction with biotin) of M6 were determined in real time using the surface plasmon resonance-based BIAcoreX biosensor (Fig. 3). Biotin-conjugated bovine serum albumin immobilized on a CM5 sensor chip was used to study the reversibility of biotin binding.[5]

Disulfide bond formation between M6 and SAK-CPFB

Purified M6 and SAK-CPFB were reduced separately by tris(2-carboxyethyl)phosphine (TCEP, Sigma) (final concentration: 2 m*M* in 10 m*M* sodium phosphate, pH 6.8, 10 m*M* sodium chloride) for 1 h at room temperature. The two protein samples were then mixed and dialyzed overnight against the same buffer without TCEP at 4°C. The total volume of the reaction mix was 100 µL. M6 was used at ~2 µg.

Surface-enhanced laser desorption/ ionization time-of-flight mass spectrometry (SELDI-TOF-MS)

Protein masses of the M6/SAK-CPFB mixtures were analyzed on a Protein Biosystem II ProteinChip Reader (Ciphergen Biosystems).[19] Chip arrays (NP20 chip) were prepared according to the manufacturer's instructions. The ProteinChip Reader was calibrated using cytochrome C (horse, 12,360.2), myoglobin (horse muscle, 16,951.5), glyceraldehyde 3-phosphate dehydrogenase (rabbit, 35,688.0) and albumin (bovine serum, 66,433.0) in the All-in-1 protein standard (Ciphergen Biosystems) with sinapinic acid as the matrix.

Crystallization and structure determination of the streptavidin-peptide complex

Lyophilized recombinant streptavidin (Sigma) was dissolved in 10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA (TE) at 8 mg/mL. Lyophilized synthetic AviTag 2 peptide (Ac-NDIFEAQ-(Biotinylated Lys)-IEYHES-NH_2) (Anaspec) was dissolved in TE at 5 mg/mL. Streptavidin (8 µL) was added to peptide (2 µL) at an approximate ratio of 1 streptavidin protomer: 1 peptide and mixed on ice for 20 min before crystallization. Crystals were grown by the hanging-drop vapour diffusion method by combining 2 µL of the protein: peptide mixture with 2 µL of precipitant solution (1.1 *M* ammonium sulphate, 0.1 *M* sodium acetate pH

	-12	-10	-8	-6	-4	-2	0	+2	+4	+6
PFB tag	G	S	G	S	L	H	H	I	L	D	A	Q	K	M	V	W	N	H	R	
AviTag							G	L	N	D	I	F	E	A	Q	K	I	E	W	H	E
AviTag 2							N	D	I	F	E	A	Q	K	I	E	Y	H	E	S	

Figure 2
Sequences of the biotinylation tags (PFB, AviTag and AviTag 2). PFB (peptide for biotinylation) and AviTag are biotinylation tags that can be efficiently biotinylated by *E. coli* biotin ligase. + or − sign above PFB refers to the position of the amino acid residue relative to the lysine residue in the tag.

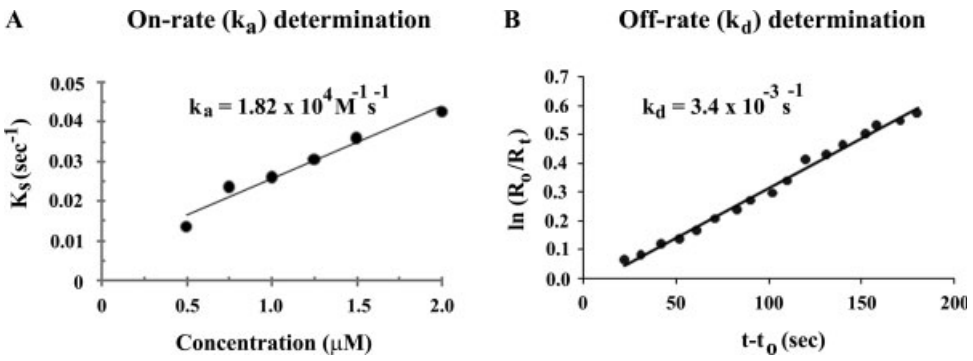

Figure 3

Kinetic parameters for the interaction between M6 and biotin as monitored by BIAcore biosensor. (**A**) On-rate (k_a) determination. k_a is derived from association analysis using a plot of k_s versus protein concentration. (**B**) Off-rate (k_d) determination. k_d is the slope of the plot of $\ln(R_0/R_t)$ versus $(t - t_0)$ where R_o is the response at an arbitrary starting time t_0; R_t is the response at time t.

3.8, 10% glycerol) and equilibrating against 1 mL of precipitant solution. Crystals appeared during 1–4 days. A crystal (0.2 × 0.1 × 0.05 mm) was soaked in 10 μL of cryosolvent (0.5 mg/mL peptide, 1.3 *M* ammonium sulphate, 20% glycerol, 200 m*M* sodium acetate pH 3.8) for 16 h before data collection. The crystal was flash-cooled in a nitrogen gas stream at 100 K (Oxford Cryostream). X-ray diffraction was measured using a RU-H3R generator (Rigaku) and Mar345 image plate detector (MarResearch) (resolution = 20–1.8 Å, R_{sym} = 0.07, redundancy = 4.5). Data were processed using the HKL suite and programs from the CCP4 package.[20,21] Because the crystal was isomorphous to the streptavidin:biotin complex (PDB 1MK5),[22] this model was used as a starting model for rigid body, positional and temperature factor refinement using Refmac (v. 5.1.24) (resolution = 20–1.9 Å, final R_{work} = 0.18, R_{free} = 0.23).[23]

RESULTS

Modeling of the biotinylated peptide-streptavidin complex

To explore the possibility to introduce cysteine residues in both the monomeric streptavidin M4 and the biotinylation tag, a model of the biotinylated peptide-streptavidin complex was generated, using the crystal structure of streptavidin-BCAP (biotinyl-ε-aminocaproic acid, an analog of biotinylated lysine) as a starting point.[11] Since all four mutated residues in M4 are located on the β strands and are known to have a strong preference to adopt β-strand secondary structure, M4 is assumed to maintain a three-dimensional, β-barrel structure similar to the natural streptavidin subunit.[24,25] A nonapeptide with lysine at the central position flanked by four serine residues at both the N- and C-terminal ends was modeled in a fully extended β-strand configuration. Superimposition of the lysine residue in this modeled peptide with the biotinyl-ε-aminocaproic acid in streptavidin-BCAP was used to generate an initial model of the biotinylated peptide-streptavidin complex. By varying the torsion angles in the lysine side chain to sterically reasonable values, it was possible to generate six reasonable models of the streptavidin-peptide complex in which the peptide adopts differing rotational orientations relative to streptavidin. Model 6 is shown in Figure 1(B).

Identification of residues in streptavidin and biotinylated peptide for replacement with cysteine

Formation of disulfide bonds has specific steric requirements [Fig. 1(A)]. The two sulfur atoms in the cysteine residues should be positioned with a bonding distance of ~2.04 Å whereas the $C_\beta—S_\gamma—S_\gamma$ angle should be ~104°.[26] To identify residues in streptavidin and the biotinylated peptide that could be replaced by cysteine and lead to the formation of an intermolecular disulfide bond with ideal geometric properties, the "Disulfide by Design" program[26] was used. The best candidate with the lowest estimated energy value (3 kcal/mol) was to replace Asn-118 in M4 and a serine residue that is located two residues away (designated − for upstream and + for downstream) from the biotinylated lysine (designated 0) in the peptide tag [Figs. 1(B) and 2) with cysteine. Models of the biotinylated peptide-streptavidin complex suggested that the cysteine residue in the biotinylated tag could be at either the +2 or −2 position. Therefore, both versions of CPFB tags were generated (see Fig. 2). At least one of these substitutions should allow the formation of an intermolecular disulfide bond [Fig. 1(C)].

Production and characterization of M6 and SAK-CPFB (±2)

To produce the desired streptavidin (M6), two extra mutations were introduced to the structural gene encoding M4. In addition to the substitution of Asn-118 by a cysteine residue (N118C), Trp-120 was replaced by serine (W120S). In the tetrameric streptavidin, Trp-120 in one subunit interacts with the neighboring subunit and buries itself at the interface. For monomeric M4, Trp-120 is likely to be solvent-exposed. The W120S mutation was designed to display a hydrophilic (Ser) rather than a hydrophobic (Trp) residue on the M6 surface to minimize nonspecific aggregation. M6 was produced by secretion from *Bacillus subtilis* and affinity purified. The majority of the purified M6 migrated as a single band using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight of ~21,000 (Fig. 4(A), lane 4). The mature monomeric M6 has an expected molecular weight of 16,482[19] which is in close agreement with the molecular weight [16,514, Fig. 4(D), peak 2] determined by SELDI-TOF mass spectrometry. Disulfide bonded dimeric M6 [Fig. 4, band (b)] could be detected at low levels in the absence of reducing agent. This band disappeared when the protein sample was treated with mercaptoethanol before loading [Fig. 4(A), lane 2]. As a model for CPFB, the C-terminal PFB tag in the staphylokinase-PFB (SAK-PFB) fusion was mutated at either the +2 or −2 position to generate SAK-CPFB (±2), respectively. These two proteins were also produced by secretion from *B. subtilis*, chromatographically purified and biotinylated *in vitro* for interaction studies. Since these two proteins were found to interact equally well with M6 (detailed analyses were present in Fig. 5), SAK-CPFB (+2) was selected as a representative for further detailed analyses and to establish the optimized conditions for M6/SAK-CPFB interactions. The biotinylated SAK-CPFB (+2) had an apparent molecular weight of 22,000 as analyzed by SDS-PAGE [Fig. 4(A), lane 5]. SELDI-TOF MS analysis confirmed its molecular weight to be ~19,115 [Fig. 4(D), peaks 1 and 4] which correlated well with the expected value of 19,093. Low levels of disulfide bonded SAK-CPFB (+2) dimer could be detected [Fig. 4(A), lane 5, band (a)] if the sample was not treated with mercaptoethanol before loading.

M6 has similar biotin binding properties as M4

With the introduction of the two mutations (N118C and W120S) to M4 to generate M6, it is vital to examine whether M6 retains similar biotin binding properties as M4 or not. With biotinylated BSA immobilized to a BIAcore CM5 sensor chip, interactions between M6 and biotin was examined in real time using BIAcore biosensor. On the basis of three independent trials for on-rate (k_a) and off-rate (k_d) determinations, M6-biotin interactions showed an average on-rate of $(1.83 \pm 0.03) \times 10^4$ M^{-1} s^{-1} and an average off-rate of $(3.41 \pm 0.07) \times 10^{-3}$ s^{-1} (see Fig. 3). The average dissociation constant (K_d) for this interaction is $(1.86 \pm 0.01) \times 10^{-7}$ *M*. These parameters are similar to those of M4 (k_a: 1.80×10^4 M^{-1} s^{-1}; k_d: 3.39×10^{-3} s^{-1}; K_d: 1.87×10^{-7} *M*)[5]. These analyses confirmed that the extra mutations introduced to M6 did not affect the kinetic parameters for the M6-biotin interaction.

Optimization of conditions for M6/SAK-CPFB(+2) heterodimer formation and the analysis of the covalent complexes

Since low levels of disulfide-bonded homodimeric M6 and SAK-CPFB (+2) complexes were observed, both samples were pretreated with TCEP, a potent reducing agent,[27] to break existing disulfide bonds before mixing the two proteins together. To optimize the formation of the M6/SAK-CPFB (+2) heterodimer, the concentration of M6 in the reaction was kept constant while the concentration of SAK-CPFB (+2) was varied systematically to generate a range of SAK-CPFB/M6 ratio from 0.5 to 4. The formation of covalent complexes was monitored by SDS-PAGE (±mercaptoethanol) and SELDI-TOF MS. As shown in Figure 4(A) (lanes 6–10), an extra dominant protein band (c) with the apparent molecular weight of 41,000 appeared if the samples were analyzed in the absence of mercaptoethanol. This band could be detected with antibodies specifically against either streptavidin or SAK [Fig. 4, panels (B,C) lanes 6–10]. Its molecular weight was determined by MS to be ~35,414 [Fig. 4(D), peaks 3 and 6] which agreed closely with the expected (35,557) molecular weight for the M6/SAK-CPFB (+2) heterodimeric complex. This band disappeared if the samples were analyzed in the presence of mercaptoethanol [Fig. 4, panels (A,B,C) lane 2]. All these data indicated that band c is the protein band for the covalently linked SAK-CPFB/M6 complex. The intensity of band c reached a plateau when the SAK-CPFB/M6 ratio was 2 or higher. This observation suggested that a ratio of 2 was the best to promote heterodimer formation. This ratio was used for all the M6/SAK-CPFB interaction studies including the SELDI-TOF MS analysis. It is unexpected that the SAK-CPFB/M6 ratio at 1 was not the best ratio for heterodimer formation. At this ratio, it seems that there was not enough SAK-CPFB to kinetically drive the heterodimer formation in a speed that could out compete the homodimer formation processes for both M6 and SAK-CPFB. Low levels of covalently linked M6 homodimers [Fig. 4(B), lane 7] and SAK-CPFB homodimers [Fig. 4(C), lane 7] were still observed under this condition. Once the M6 homodimers were formed, they could no longer be able to form the stable covalent heterodimeric complexes with SAK-CPFB and vice versa. In contrast, no M6 homodimers could be detected when the

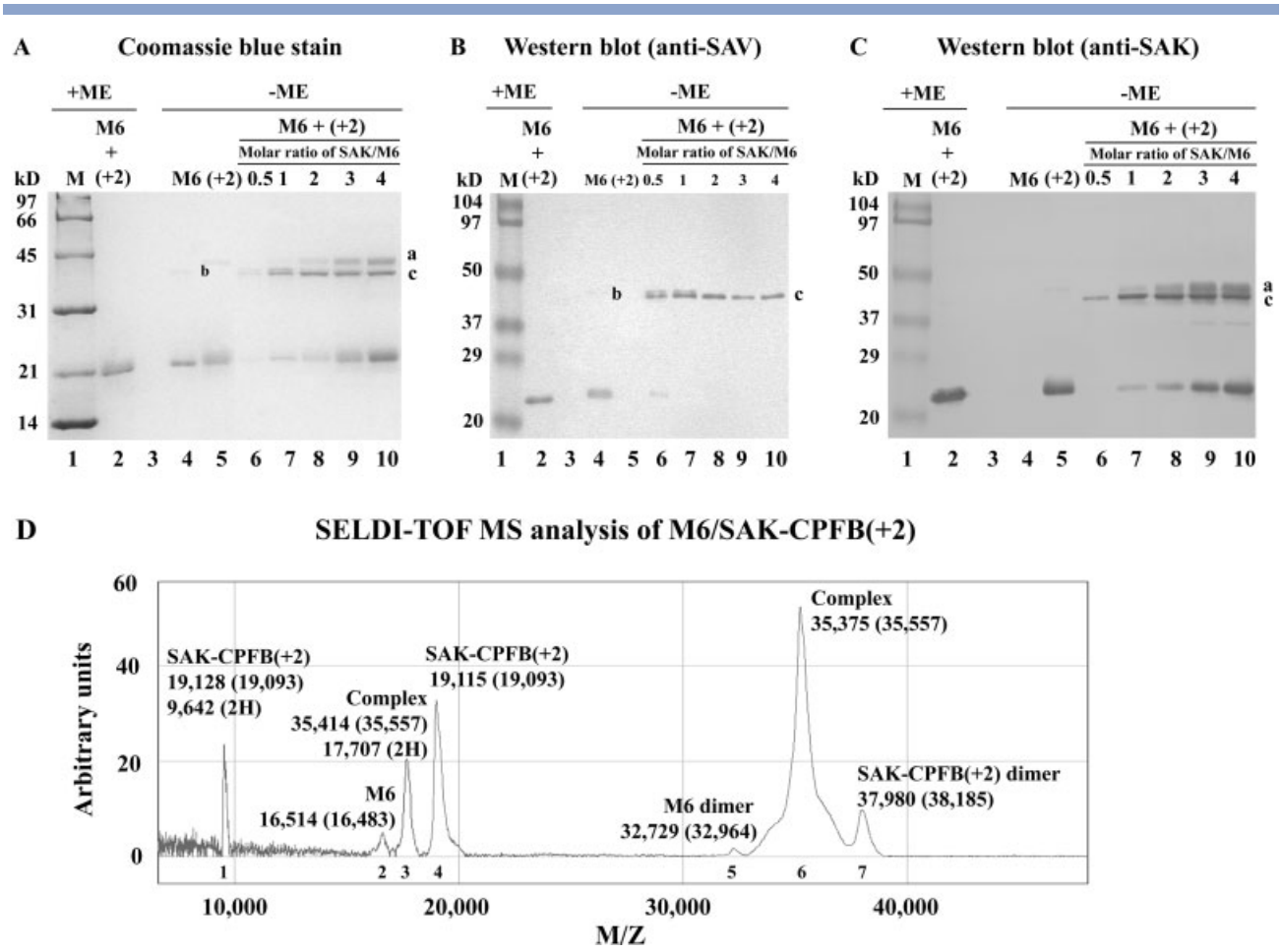

Figure 4

Disulfide bond formation between M6 and SAK-CPFB (+2). Reduced M6 and biotinylated SAK-CPFB (+2) (cysteine at the +2 position) were mixed using varying amounts of SAK-CPFB. The amount of M6 in the reaction mix was kept constant while the molar ratio of SAK-CPFB:M6 was increased from 0.5:1 to 4:1. Proteins in the samples were analyzed by SDS-PAGE (Panel **A**) stained by coomassie blue and Western blotting [Panels **B** (probed with streptavidin specific antibodies) and **C** (probed with staphylokinase specific antibodies)]. +ME and −ME indicate the presence and absence of mercaptoethanol in the sample loading buffer. a, b and c represent the disulfide bonded homodimeric SAK-CPFB with an apparent molecular mass of 44 kDa, the disulfide bonded homodimeric M6 with an apparent molecular mass of 42 kDa and the disulfide bonded heteroduplex of SAk-CPFB/M6 with an apparent molecular mass of 41 kDa, respectively. Lane numbers are shown at the bottom of the gel or the blots. Protein molecular weight markers and the prestained markers are in lane 1 marked with M. Lane 3 represents an empty lane to avoid the spill of mercaptothanol from the mercaptothanol containing samples to the mercaptoethanol-free samples. Panel (**D**) shows the SELDI-TOF mass spectrometry analysis of protein mixture formed by mixing SAK-CPFB (+2) and M6 at a ratio of 2:1. Seven peaks are marked. Two of these peaks (Peaks 1 and 3) carry two positive charges. The determinted molecular weights of the proteins (or protein complexes) are shown with the expected molecular weights shown in bracket. Both peaks 3 and 6 represent the disulfide bonded M6/SAK-CPFB(+2) complex.

SAK-CPFB/M6 ratio was 2 or higher. Under these conditions, presence of SAK-CPFB (+2) in both the monomeric and homodimeric states [Fig. 4, band (a)] in significant amounts was observed as SAK-CPFB (+2) was in excess.

Rotation of the biotinylated PFB peptide tag in the streptavidin-biotinylated PFB complex

Since M6 could form a covalent heterodimeric complex with SAK-CPFB (+2), it is of great interest to see whether M6 can form a similar covalent complex with SAK-CPFB (−2). Observation with four independent experiments showed that both SAK-CPFB (±2) formed covalent complexes with M6 with equal efficiency at a SAK-CPFB/M6 ratio of 2. One set of the typical data was shown in Figure 5 (Panels A, B, and C). Formation of the covalent M6/SAK-CPFB heterodimer was biotin dependent. When SAK-CPFB (±2) molecules were not enzymatically biotinylated, no heterodimer could be observed [Fig. 5(D), lanes 4 and 5). This indicates that once the biotin moiety in the biotinylated CPFB is captured by M6, the CPFB tag can rotate on the surface of

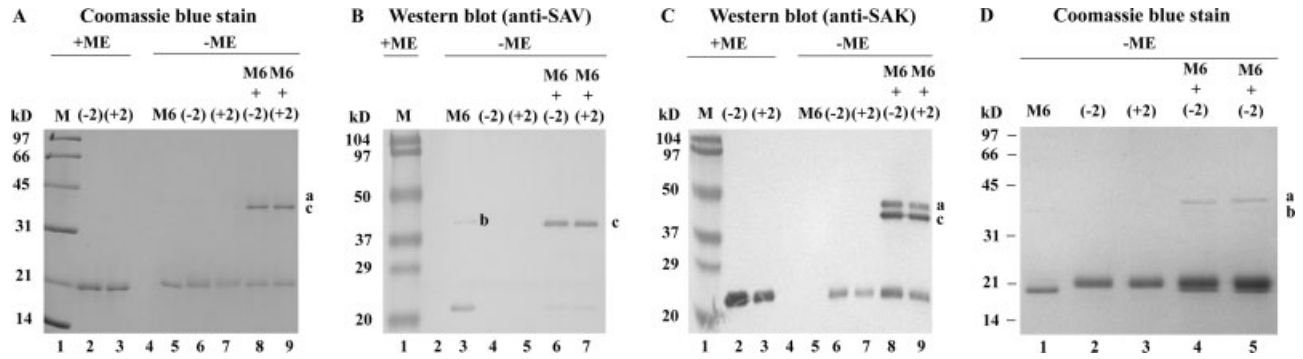

Figure 5

Formation of M6/SAK-CPFB (+2) and M6/SAK-CPFB (−2) in a biotinylation dependent manner. SAK-CPFB and M6 and were mixed at a molar ratio of 2:1. SAK-CPFB samples shown in panels **A–C** were all enzymatically biotinylated. The numbers −2 and +2 represent the SAK-CPFB protein with cysteine at the −2 and +2 position, respectively. (a) disulfide bonded SAK-CPFB dimers; (b) disulfide bonded monomeric streptavidin M6 dimers; and (c) disulfide bonded heteroduplex of M6 and biotinylated SAK-CPFB; +ME: samples were treated with β-mercaptoethanol; −ME: samples were not treated with β-mercaptoethanol, M: molecular weight markers. (A) Samples were analyzed by SDS-PAGE with the gel stained by Coomassie blue. In lanes 2 and 3, the upper band is SAK-CPFB and the lower band is M6, (B) Western blot probed with streptavidin (SAV) specific antibodies, (C) Western blot probed with staphylokinase (SAK) specific antibodies, (D) Samples were analyzed by SDS-PAGE with the gel stained by Coomassie blue. These samples are similar to those shown in panels A–C except that the SAK-CPFB samples used were nonbiotinylated.

the complex with the biotinylated lysine as the axis until the formation of a disulfide bond. As shown in Figure 1(C), enzymatic biotinylation links the carboxylic group in biotin to the ε-amino group in the lysine side-chain. At least four torsion angles in the lysine side chain can adopt a wide range of torsion angles to allow multiple rotational orientations of the biotinylated peptide relative to streptavidin. Consequently, SAK-CPFB (±2) should be able to form disulfide bonds with cysteine fixed at position 118 of M6.

Flexibility of the cysteine position within the biotinylated tag for formation of M6-biotinylated PFB complexes

To explore the possibility to introduce a cysteine residue at locations other than the ±2 positions in the CPFB tag, six new tags at the C-terminal end of SAK were generated with the cysteine residue located at either position ±1, +4, −6, −10 or position −12 (see Fig. 2). As biotinylation efficiency for CPFB (±1) was very low, the experiment was not conducted on these tags. Surprisingly, cysteine at any of the aforementioned positions was found to be able to form disulfide bonds with M6, although the efficiency of disulfide bond formation was found to be significantly lower with CPFB (−12) (data not shown). These findings have two implications. First, cysteine can be positioned on either the + or − side of the biotinylated lysine residue in the tag. This observation strengthens the idea that the CPFB tag can rotate on the surface of the M6/SAK-CPFB complex until the formation of the disulfide bond. Second, cysteine residues located at positions further away from positions ±2 in the CPFB tag can fold back and react with C-118 of M6. This indicates the dynamic nature and flexibility of the C-terminal end of CPFB when bound to SAK.

Structure determination of the streptavidin/AviTag 2 complex

An attempt was made to experimentally determine the structure of the streptavidin-CPFB complex using X-ray crystallography. Rotation of the CPFB tag in the covalent complex was expected to induce disorder in the structure and impair the visualization of the PFB portion of the SAK-CPFB complex. In an attempt to reduce the presence of disorder, a new 14 amino-acid tag (AviTag 2) was designed using the model shown in Figure 1(B). AviTag 2 was modified from AviTag, a PFB tag derivative, by replacing W in +2 position of the avitag to Y and the addition of a serine residue at position +6 (see Fig. 2). The idea was to introduce some putative hydrogen bonding between the tag and the surface of M6 so as to fix the orientation of AviTag 2 in the complex. Biotinylated AviTag 2 was chemically synthesized using biotinylated lysine instead of lysine and HPLC purified. Although crystals of the complex clearly revealed the structure of streptavidin and the biotinylated lysine residue of the bound peptide, the remaining portions of the peptide were not defined by electron density, presumably because of the presence of disorder. This observation again suggests that the bound biotinylated peptide can adopt multiple rotational orientations relative to streptavidin.

DISCUSSION

In this study, interactions between M6 and CPFB containing proteins are suggested to go through a two-stage

process (capture and immobilization) to generate the covalently linked heterodimeric complex. Biotinylation is essential in the capture stage [Fig. 5(D)]. This capture step can increase the effective concentration of the two interacting cysteine residues by restricting the motion of the biotinylated SAK-CPFB (±2) to the surface of M6 around the biotin binding site. Following this initial capture step, the subsequent formation of a disulfide bond results in the immobilization of SAK-CPFB (±2) to the surface of M6.

This study demonstrates that a substantial amount of flexibility is allowed in the design of the CPFB tag. First, replacement of a residue in the PFB tag does not significantly affect the biotinylation efficiency in most tested cases. Out of eight positions tested, only positions ±1 were not acceptable. Second, the cysteine residue can reside either upstream or downstream from the biotinylated lysine in the peptide tag. This is made possible by the fact that the biotinylated CPFB can rotate on the M6 surface with biotin bound to the biotin binding pocket in M6 before the formation of the disulfide bond. Finally, some flexibility is allowed regarding the position in the tag for introducing the cysteine residue. Although molecular modeling suggested that positions ±2 are the best choices if the peptides adopt an extended β-strand conformation, the ability of the CPFB tag to adopt other conformations or to fold back to M6 allows the cysteine residue to be located at positions that are farther away without impairing the formation of disulfide bonds.

To make this M6/SAK-CPFB system to work, preparation of M6 in its fully functional form is essential. Initially, M6 was prepared using the protocol developed for M4. A portion (~10%) of purified M6 was found to be in the aggregated state as analyzed by a size exclusion chromatography system equipped with on-line dynamic and static light scattering detectors. The aggregated M6 was inactive and, consequently, was not able to form heterodimeric complexes with biotinylated SAK-CPFB even when the ratio of SAK-CPFB to M6 is higher than 2. This was evidenced by the presence of a M6 protein band migrated in the monomeric state in SDS-PAGE. Our design for the generation of monomeric streptavidin mainly relies on the introduction of positive-charge repulsion at the subunit interfaces created by the T76R and V125R mutations in M4 and M6. Therefore, a rational approach to minimize M6 aggregation is to lower the ionic strength in the buffer so as to maximize the charge repulsion of M6. Indeed, the replacement of physiological buffered saline (PBS which contains 150 m*M* NaCl) in the purification protocol with a low salt buffer containing 10 m*M* NaCl significantly improved the situation. Most of the M6 proteins purified under this condition were active and could be driven to form the covalently linked heterodimeric complex in the presence of excess SAK-CPFB with a ratio of SAK-CPFB to M6 at 2 or higher (Figs. 4 and 5).

The engineered monomeric streptavidin M6 and SAK-CPFB (±2) system offers infinite binding affinity via covalent bond formation between M6 and CPFB. Because the covalent bond is a disulfide bond which can be easily disrupted under one's control, M6 exhibits the same immobilization capability as the natural tetrameric streptavidin but at the same time retains the reversibility for bound ligands as seen with monomeric streptavidin. By combining the advantages of both tetrameric and monomeric streptavidin in M6, this engineered protein is a novel biomaterial with many potential applications. One application could be the development of reversible or reusable immobilization devices (e.g. bioreactor, sensor and protein chips). The principle demonstrated here can potentially be applied to recombinant monomeric avidin[28] and other protein–protein/peptide interaction systems such as streptavidin-Streptag.[29] Recently, a monovalent streptavidin with high biotin binding affinity has been elegantly developed.[30] It is a tetrameric streptavidin with only one active biotin binding site per tetramer. This system has been successfully applied to quantify biotinylated recombinant cell surface receptor-biotinylation tag fusions without the concern of streptavidin-induced crosslinking of the receptors. Similar applications can also be applied using the M6-CPFB system. With the recent interest in the development of synthetic biology[31] which focuses on the design and redesign of new or pre-existing biological materials for novel applications, the M6-CPFB system can serve as a building block (or biobrick) for the engineering of the more sophisticated biological materials.

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